



Cavallo, D., Landucci, E., Gerace, E., Lana, D., Ugolini, F., Henley, J. M., Giovannini, M. G., & Pellegrini-Giampietro, D. E. (2019). Neuroprotective effects of mGluR5 activation through the PI3K/Akt pathway and the molecular switch of AMPA receptors. *Neuropharmacology*, 162, [107810].
<https://doi.org/10.1016/j.neuropharm.2019.107810>

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[10.1016/j.neuropharm.2019.107810](https://doi.org/10.1016/j.neuropharm.2019.107810)

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Neuroprotective effects of mGluR5 activation through the PI3K/Akt pathway and the molecular switch of AMPA receptors

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Highlights

- mGluR5 activation with DHPG and VU0092273 showed neuroprotective effects in OGD.
- mGluR5 exerted its neuroprotective effects via PI3K/Akt pathway.

- mGluR5 also prevented the GluA2 down-regulation following an ischemic injury.
- mGluR5 appears to stimulate neuroprotective responses in astrocytes and microglia.

Abstract

Previous studies have demonstrated that antagonists of mGluR1, but not mGluR5, are neuroprotective in models of cerebral ischemia. To investigate the individual roles of mGlu1 and mGlu5 receptors in *in vitro* model of cerebral ischemia we used low doses of the non-selective group I agonist DHPG and mGlu1 and mGlu5 selective positive allosteric modulators (PAMs). In hippocampal slices subjected to 30 minutes oxygen-glucose deprivation (OGD), DHPG (1 μ M) and the mGluR5 PAM (VU0092273) significantly reduced OGD-induced CA1 injury monitored by propidium iodide staining of the slices and quantitative analysis of CA1 neurons. In contrast, the mGluR1 PAM (VU0483605) showed no neuroprotection. These protective effects of DHPG and VU0092273 were prevented by inhibition of PI3K/Akt pathway by LY294002. The mGluR5 PAM (VU0092273) also prevented GluA2 down-regulation triggered by ischemic injury, via PI3K/Akt pathway, revealing a further contribution to its neuroprotective effects by reducing the excitotoxic effects of increased Ca^{2+} influx through GluA2-lacking AMPA receptors. Furthermore, immunohistochemical assays confirmed the neuroprotective effect of VU0092273 and revealed activation of glia, indicating the involvement reactive astrogliosis in the mechanisms of neuroprotection. Our data suggest that selective activation/potentialiation of mGlu5 signalling represents a promising strategy for the development of new interventions to reduce or prevent ischemia-induced neuronal death.

Keywords: metabotropic glutamate receptors; Positive Allosteric Modulators (PAMs); PI3K/Akt; GluA2 subunit; astrocytes; microglia; oxygen-glucose deprivation (OGD).

1. Introduction

Ischemia caused by stroke is one of the main causes of mortality and long-term disability worldwide (Law et al., 2017). Massive glutamate release following an ischemic injury triggers an excitotoxic cascade, which leads to glutamate receptor-mediated delayed cell death of vulnerable neuronal populations (Buchan & Pulsinelli, 1990).

Metabotropic glutamate (mGlu) receptors are classified into three subgroups (Groups I, II and III) based on sequence homology, signalling transduction pathways and relative pharmacology (Conn & Pin, 1997). Of these, group I comprises mGlu1 and mGlu5, which coupled to $G_{q/11}$ and mediate $\text{IP}_3/\text{Ca}^{2+}$ signal transduction via phospholipase C (PLC) activation (Abe et al., 1992). Although the two subtypes of group I metabotropic glutamate receptors are highly homologous they play

different roles in the cerebral ischemia. mGlu1, but not mGlu5, antagonists are neuroprotective in models of cerebral ischemia (Pellegrini-Giampietro, 2003). In addition, mGlu5 is expressed on astrocytes where it contributes to the modulation of synaptic transmission efficacy and local blood flow (Arizono et al., 2014) and on microglia where it mediates numerous actions essential for intrinsic glial cell function, as well as for glial–neuronal interactions (Loane et al., 2012).

Positive allosteric modulators, PAMs, are compounds that interact at “allosteric” sites, topographically distinct from the orthosteric site, where they can affect the affinity and/or efficacy of an orthosteric ligand, a property referred to as cooperativity. This is useful because the allosteric site contains a higher level of sequence diversity between receptor subtypes than the orthosteric site, allowing for greater subtype selectivity of allosteric ligands (Christopoulos and Kenakin, 2002; Conn et al., 2009). Moreover, allosteric agents have the potential to show differential effects on downstream signalling pathways, termed functional selectivity. Thus, PAMs provide a potential opportunity to develop agents that selectively target GPCR signalling pathways critical for therapeutic efficacy without modulating signalling pathways that lead to adverse effects (Nickols & Conn, 2013).

To better discriminate the role of group I metabotropic glutamate receptors we used the non-selective agonist DHPG and two selective positive allosteric modulators (PAMs), mGluR1 PAM 3-chloro-N-(3-chloro-4-(4-chloro-1,3-dioxoindolin-2-yl)picolinamide, VU0483605, and the mGluR5 PAM (4-hidroxypiperidin-1-yl)(4-(phenylethynyl)phenyl)methanone, VU0092273, in the in vitro model of OGD. Our results show that selectively enhancing mGluR5 signalling can be neuroprotective against neuronal death in CA1 via a mechanism that involves both activation of the PI3K/Akt pathway and the prevention of the GluA2 AMPA subunit down-regulation induced by OGD.

2. Materials and methods

Experiments and animal use procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). The experimental protocols were approved by the Animal Committee of the Department of Pharmacology, University of Florence, in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No. 123) and the European Communities Council Directive of 24 November 1986 (86/609/EEC). The authors further attest that all efforts were made to minimize the number of animals used and their suffering.

2.1. Materials

(S)-3,5-Dihydroxyphenylglycine (DHPG), (S)-(+)- α -amino-4-carboxy-2-methylbenzeneacetic acid (LY367385) and 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) were purchased

from Tocris Cookson (Bristol, UK). 1-Naphthylacetyl spermine trihydrochloride (NASPM) was purchased from Sigma-Aldrich, St. Louis, MO, USA). 3-Chloro-N-[3-chloro-4-(4-chloro-1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)phenyl]-2-pyridinecarboxamide (VU0483605) and (4-hydroxypiperidin-1-yl)-[4-(2-phenylethynyl)phenyl]methanone (VU0092273) were generously provided by Dr. Jeffrey P. Conn (Vanderbilt University Medical Center, Nashville, TN, USA). 2-(4-Morpholinyl)-8-(4-aminophenyl)-4H-1-benzopyrone-4-one (LY 294002) was purchased from Calbiochem (Merck Biosciences Ltd, Nottingham, UK). Propidium iodide (PI) was purchased from Molecular Probes (Leiden, the Netherlands) and tissue culture reagents were obtained from Gibco-BRL (San Giuliano Milanese, MI, Italy) and Sigma (St Louis, MO, USA).

2.2. Oxygen-Glucose Deprivation in Organotypic Hippocampal Slice Cultures

Organotypic hippocampal slice cultures were prepared as previously reported in detail ([Gerace et al., 2012](#)). Briefly, hippocampi were removed from the brains of 7-9 days old Wistar rats (Harlan, MI, Italy), and transverse slices (420 μ m) were prepared using a McIlwain tissue chopper. Slices were then transferred onto 30 mm diameter semiporous membranes inserts (Millicell-CM PIC M 03050; Millipore, Italy), which were placed in six-well tissue culture plates containing 1.2 ml culture medium per well. Slices were maintained at 37°C in an incubator in atmosphere of humidified air and 5% CO₂ and the culture medium was changed thrice a week. Experiments were carried out after 14 days in vitro. The slices were subjected to OGD by exposing them to a serum- and glucose-free medium saturated with 95% N₂ and 5% CO₂ at 37°C in an airtight anoxic chamber equipped with an oxygen gas controller (BioSpherix, New York, USA). After 30 min, the cultures were transferred to oxygenated serum-free medium containing 5 mg/ml glucose and maintained at 37°C, 35°C or 32°C in different incubators under normoxic conditions until neuronal injury was evaluated 24 h later. Drugs were present in the incubation medium during OGD and the subsequent 24 h recovery period (Fig. 1A). Cell injury was assessed by using the fluorescent dye PI (5 μ g/ml); fluorescence was viewed using an inverted fluorescence microscope. Images were digitized using a video image controlled by software and subsequently analysed using a morphometric analysis software. In order to quantify cell death, the CA1 hippocampal subfield was identified and encompassed in a frame using the drawing function in the image software (ImageJ; NIH, Bethesda, USA) and the optical density of PI fluorescence was detected. Control experiments performed in our laboratory, as well as a number of previous studies using organotypic hippocampal slices exposed to ischemia-related insults, have shown that there is a linear correlation between relative PI fluorescence and the number of injured cells as detected by morphological criteria ([Gerace et al., 2012](#)).

2.5. Western blot analysis

Hippocampal slices were washed with cold 0.01 M phosphate-buffered saline, pH 7.4 and 4 slices/sample were gently transferred and dissolved in a tube containing 1% sodium dodecyl sulfate (SDS) as previously reported in detail (Gerace et al., 2016; Landucci et al., 2016). Total protein levels were quantified using the Pierce (Rockford, IL, USA) BCA (bicinchoninic acid) Protein Assay. Homogenates (40 µg of protein) were resolved by electrophoresis on 4–20% (wt/vol) SDS-polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto nitrocellulose membranes using the transblot TURBO (Bio-Rad, Hercules, CA, USA). Blots were blocked for 1 h at room temperature in Tris-buffered saline, pH 7.6, 0.1% Tween 20 (TBS-T) containing 5% non-fat dry milk, and then incubated overnight at 4°C with a polyclonal rabbit antibody directed against phospho-Akt (Ser473) or phospho-GSK3-β (Ser9) (all from Cell Signaling Technology, Beverly, MA, USA) diluted 1:1000 in TBS-T containing 5% bovine serum albumin and polyclonal goat anti-GluA2 (Santa Cruz Biotechnology, Paso Robles, CA, USA) diluted 1:1000 in TBS-T containing non-fat dry milk. Monoclonal α-tubulin antibody (Sigma, 1:5000) was used as loading control. Immunodetection was performed with secondary antibodies (1:2000 anti-rabbit, anti-goat and anti-mouse IgG from donkey; Amersham Biosciences, Buckinghamshire, UK) conjugated to horseradish peroxidase. The reactive bands were detected using chemiluminescence (ECLplus; Euroclone, Padova, Italy). Quantitative analysis was performed using the QuantityOne analysis software (Bio-Rad, Hercules, CA, USA).

2.6. Triple labelling immunohistochemistry

Organotypic hippocampal slices were triple immunostained with the free floating method as described (Lana et al., 2014). Briefly, after blocking with Blocking Buffer (BB), slices were incubated overnight under agitation with the following primary antibodies: a mouse monoclonal anti-neuronal nuclei (NeuN, 1:200, Millipore, Billerica, MA, USA) for neurons, and a rabbit polyclonal anti IBA1, (1:300, WAKO, Osaka, Japan) for microglia, both diluted in BB solution. IBA1 (Ionized calcium binding adaptor molecule 1) is an intracellular 17-kDa protein whose expression is restricted to microglia/macrophages (Imai et al., 1996) Iba1 is a protein for cytoskeletal rearrangement in microglia, widely used as a specific marker for total microglia. All the following steps were done at room temperature in the dark for 2 h. After washings, slices were incubated with an anti-rabbit secondary antibody conjugated with AlexaFluor 635 (for microglia, 1:400, Thermo Fisher Sci., Waltham, MA, USA). After incubation, a secondary antibody conjugated with AlexaFluor 555 (for neurons, 1:400, Thermo Fisher Sci.) was added, and slices were incubated as above. Finally, astrocytes were immunostained with a primary monoclonal anti GFAP antibody conjugated with AlexaFluor 488 (1:500, Millipore). Glial Fibrillary Acidic Protein (GFAP), is a structural protein (Bignami et al., 1972) a key component of the astrocyte's cytoskeleton, and classically used as a marker for astrocytes (Middelorp and Hol, 2011). After washings, the slices

were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA).

2.7. Confocal laser scanning microscopy and quantitative analysis

Images were acquired under a LEICA TCS SP5 confocal laser scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) with a 20X objective. Confocal scans were taken at 1 μ m z-steps keeping all parameters (pinhole, contrast and brightness) constant. Image analyses were conducted on z-stacks which contained the region of interest, corresponding to the CA1 area. Images were digitally converted to green, red or blue using Image J (National Institute of Health, <http://rsb.info.nih.gov/ij>).

Quantification of astrocytes, neurons and microglia immunofluorescence was performed separately using Image J. Three consecutive z-scans (1 μ m each, total 3 μ m) were stacked, starting at 10 μ m inside the slice and an appropriate and constant threshold level was selected. Quantitation of immunofluorescence of NeuN, GFAP or IBA1 was then obtained from the ratio between positive pixels above threshold and total pixels in each region of interest.

2.10. Statistical analysis

Data are presented as means \pm SEM of n experiments. Statistical significance of differences were analysed using one-way ANOVA followed by the post hoc Dunnett and Tukey's w-test for multiple comparisons. All statistical calculations were performed using GRAPH-PAD PRISM v. 5 for Windows (GraphPad Software, San Diego, CA, USA). A probability value (P) of < 0.05 was considered significant.

3. Results

3.1. Activation of mGlu5 receptors is neuroprotective in rat organotypic hippocampal slices exposed to OGD

We have reported previously that the nonselective group I mGlu receptor agonist DHPG at 100 and 300 μ M exacerbates OGD injury in organotypic hippocampal slices ([Pellegrini-Giampietro et al., 1999](#)) and that this toxic effect is most likely mediated by mGlu1 rather than mGlu5 receptors ([Pellegrini-Giampietro, 2003](#)). On the other hand, we observed that DHPG induces ischemic tolerance at subtoxic (< 10 μ M) concentrations in the same model via the contribution of both mGlu1 and mGlu5 receptors ([Werner et al., 2007](#)). Therefore, in this study examined whether low, subtoxic concentrations of DHPG could also protect organotypic hippocampal slices against OGD injury.

Consistent with our previous observations ([Pellegrini-Giampietro et al., 1999](#); [Meli et al., 2002](#)), when relatively high concentrations of DHPG (100-300 μ M) were added to the incubation

medium of hippocampal slices during OGD and the 24 h recovery period the damage in the CA1 region induced by simulated ischemia was significantly enhanced (Fig. 1). Conversely, a low concentration of DHPG (1 μ M) produced a 30% reduction in OGD injury that was completely prevented by the mGlu5 antagonist MPEP (10 μ M) but not by the mGlu1 antagonist LY367385 (100 μ M). An intermediate concentration of DHPG (10 μ M) was neither toxic nor neuroprotective under our experimental conditions.

To corroborate the contribution of mGlu5 receptors in the protective effects of DHPG, we compared the effects of VU0483605, a positive allosteric modulator (PAM) of mGlu1 receptors with an EC₅₀ of 350 nM (Cho et al., 2014), and VU0092273, a PAM of mGlu5 receptors with an EC₅₀ of 10 nM (Noetzel et al., 2012). In hippocampal slices exposed to 30 min OGD, the mGlu1 PAM VU0483605 (1 μ M) did not reduce OGD injury when present during the 30 minutes of OGD and the subsequent 24 h recovery period. Moreover, there was no effect of the mGlu1 antagonist LY367385 (100 μ M) (Fig. 2). On the other hand, the mGlu5 PAM VU0092273 (10 nM) reduced PI fluorescence in the CA1 area by approximately 50%, and this protective effect was reversed by the mGlu5 antagonist MPEP (10 μ M) (Fig. 2).

3.2. Effects of the activation of mGlu5 receptors on neurons, astrocytes and microglia in the CA1 subregion of rat organotypic hippocampal slices exposed to OGD

We next assessed the effects of DHPG and VU0092273 on both CA1 pyramidal cell viability and astrocyte and microglia proliferation by using triple immunostaining for NeuN, GFAP and IBA1 on organotypic slices harvested 24 h after 30 min OGD, followed by confocal microscopy. Representative images are shown in Fig. 3 (red for neurons, green for astrocytes and blue for microglia) obtained from triple immunostaining experiments performed on the same slice exposed to control conditions (Fig. 3. A1-C1a), 30 min OGD (Fig. 3. A2-C2a), 30 min OGD in the presence of 1 μ M DHPG (Fig. 3. A3-C3a), and 30 min OGD in the presence of 10 nM VU009227 (Fig. 3. A4-C4a).

NeuN immunostaining (Fig. 3. A1-A4 and 3D) confirmed that viability of CA1 pyramidal neurons exposed to 30 min OGD was significantly decreased (by 32%) as compared to control slices. This loss of neurons was prevented by 1 μ M DHPG and 10 nM VU0092273. Conversely, GFAP immunostaining (Fig. 3. B1-B4 and 3E) showed that astrocytes were not significantly affected 24 h after OGD injury. Quantitative analysis (Fig. 3E) demonstrated that both treatments with DHPG (+41% vs. control) and VU0092273 (+62% vs. control) increased GFAP levels in CA1 24 h after OGD, but only the effect of VU0092273 was statistically significant. Higher resolution images of the indicated areas (Fig. 3. B1a-B4a) indicate that, after treatment with VU0092273, astrocytes were distributed in overlapping domains, showed morphological changes such as hypertrophy of the cell body and processes (Fig. B4a, open arrow), and displayed increased

expression of GFAP, conditions that resemble those defined as adaptive reactive astrocytosis (Burda & Sofroniew, 2014; Sofroniew, 2015; Choudury and Ding, 2016).

Quantitative analysis of IBA1 immunostaining (Fig. 3, C1-C4 and F) showed that the density of microglia in CA1 was not modified 24 h after OGD. Treatment with DHPG and VU0092273 significantly increased microglia proliferation by 44.5 % and 65%, respectively, in comparison to controls (Fig. 3F). The magnifications of the framed areas of panels C1-C4 (Fig. 3, C1a-C4a) show that in OGD slices treated with DHPG and VU0092273 microglia cells display a morphology typical of reactive microglia (Milligan & Watkins, 2009; Cerbai et al., 2012; Lana et al., 2014). In particular, some microglia cell bodies were enlarged and had amoeboid shape (Fig. 3, C4a, open arrow), others were rod-shaped (Fig. 3, C3a, white arrow) and their cellular branches were shorter, de-ramified and twisted. This effect was present, albeit to a lesser extent, in untreated OGD slices (Fig. 3, C2a).

3.3. The PI3K–Akt signaling pathway mediates the protective effects induced by mGlu5 receptors

Allosteric agents have the potential to exert differential effects on downstream signaling, a phenomenon termed functional selectivity (Nickols & Conn, 2014). Interestingly, we have shown previously in rat organotypic hippocampal slices that activation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway with the subsequent activation by phosphorylation of the pro-survival protein kinase Akt are implicated in the neuroprotective mechanisms of DHPG preconditioning (Scartabelli et al., 2008). We tested if the neuroprotective effects evoked by mGlu5 receptor activation with a selective PAM were mediated by this kinase signaling cascade. Figure 4A shows that the PI3K inhibitor LY294002 at 10 μ M prevented the neuroprotective effects of both 1 μ M DHPG and 10 nM VU0092273, indicating that PI3K activation is involved in the mechanisms following mGlu5 activation. To examine the downstream effectors of PI3K activation, we used phospho-specific antibodies to measure the relative levels of the phosphorylated, active form of Akt and of the phosphorylated, inactive form of GSK3- β in hippocampal slices 24 h after OGD (Figs. 4B, C). Exposure to OGD induced a marked reduction in the phosphorylation of both Akt (by 68%) and GSK3- β (by 60 %), that was completely prevented by co-incubation with the mGlu5 PAM VU0092273 (10 nM) but not the mGlu1 PAM VU0483605 (1 μ M). The PI3K inhibitor LY294002 at 10 μ M was able to revert the effects of PAM VU0092273, indicating that the mGlu5-mediated increases in the phosphorylation of Akt and GSK3- β requires activation of the PI3K-Akt cascade.

3.4. Activation of mGlu5 receptors prevents the down-regulation of the GluA2 AMPA receptor subunit induced by OGD

We have previously shown that OGD induces a down-regulation in the protein expression of the GluA2 subunit of AMPA receptors in organotypic hippocampal slices prior to CA1 pyramidal cell death (Llorente et al., 2015). This phenomenon has been designated as the AMPA receptor

'molecular switch', that is triggered by cerebral ischemia and other neurological disorders and leads to the formation of GluA2-lacking Ca^{2+} -permeable AMPA receptor channels and neurodegeneration in cells destined to die (Pellegrini-Giampietro et al., 1997; Liu et al., 2006; Henley & Wilkinson, 2016; Koszegi et al., 2017). Twelve hours after OGD, a time point preceding CA1 neuronal death, Western blot analysis confirmed that the protein expression of the crucial GluA2 subunit that confers Ca^{2+} impermeability to AMPA receptor channels, was down regulated by 41% (Fig. 5A). The formation of GluA2-lacking AMPA receptors leading to neurodegeneration in our hippocampal slices was confirmed using NASPM, a selective blocker of Ca^{2+} -permeable AMPA channels, that was able to dose-dependently attenuate the neuronal injury observed 24 h after OGD (Fig. 5B).

Because activation of group I mGlu receptors has been shown to reverse the formation of Ca^{2+} -permeable AMPA channels in cocaine-treated mice *ex vivo* (Bellone & Lüscher, 2006), we explored whether activation of mGlu receptors in our hippocampal slices could modify the changes in expression of AMPA receptor subunits observed following OGD. Interestingly however, the down-regulation of the GluA2 protein induced by OGD could be prevented only by the mGlu5 PAM VU0092273, suggesting that mGlu5 activation is responsible for the deleterious AMPA molecular switch that leads to OGD injury. We also examined whether the PI3K/Akt pathway could mediate the effects of mGlu receptor activation on the down-regulation of the GluA2 subunit induced by OGD. Interestingly, the PI3K inhibitor LY294002 (10 μM) was able to prevent the reversion of the GluA2 down-regulation induced by the mGlu5 PAM VU0092273, but not that induced by DHPG and the mGlu1 PAM VU0483605 (Fig. 5A).

4. Discussion

In this study, we show that mGlu5 exerts neuroprotective effects against OGD-induced CA1 injury in rat organotypic hippocampal slices. Using the non-selective group I mGlu agonist DHPG and the mGlu5 selective positive allosteric modulator (PAM) VU0092273 we observed significant reductions in OGD-induced CA1 injury. In contrast the mGlu1 selective PAM VU0483605 had no protective effect. Moreover, the protective actions of DHPG and VU0092273 were blocked by the mGlu5 antagonist MPEP, but not by the mGlu1 antagonist LY367385.

Immunohistochemistry revealed that DHPG and VU0092273 increased the viability of CA1 neurons exposed to OGD and enhanced glia responses, as demonstrated by the hypertrophy of astrocytes processes, and by the amoeboid-shape of microglia cell bodies. It is known that the response of astrocytes to brain injury are different in space and time and can have immediate and positive effects on cell survival or long-term delayed influence, affecting recovery. Indeed, it is still a matter of debate whether reactive astrocytes are harmful or beneficial, since both types of effects have been observed (Alilain et al., 2011; Voskuhl et al., 2009). Different subtypes of reactive astrocytes,

such as A1 and A2, differ in their functions, depending on the nature, localization, and time after injury (Liddelow and Barres, 2017). Our results suggest that mGlu5 expressed in astrocytes (Arizono et al., 2014) could stimulate their positive effects contributing to neuroprotection. Moreover, this astrocyte activation also correlates with microglial activation, a mechanism that can contribute to identification and elimination of cellular debris and neutrophils (Neumann et al., 2008; Milligan & Watkins, 2009; Cerbai et al., 2012; Lana et al., 2014) exerting a neuroprotective effect. Indeed, although microglia has long been considered a major contributor of postischemic inflammation, our data are in accordance with the growing evidence that the inflammatory response may be important for tissue repair (Madinier et al., 2009). It has been recently demonstrated that while M1-type microglia release proinflammatory and cytotoxic mediators, M2-type microglia can dampen inflammation and promote tissue regeneration (Chawla et al., 2011). These data are consistent with the concept that reactive gliosis may contribute to decrease of neuronal vulnerability (Burda & Sofroniew, 2014; Pekny & Pekna, 2014; Lana et al., 2016). mGluRs are expressed on astrocytes and microglia and their activation under pathophysiological conditions may shift microglia towards a pro-repair phenotype (Loane et al., 2012; Loane et al., 2014). Further investigations are needed to understand the mechanism underlying the mGluR5 involvement in astrocytes/microglia activation.

Positive allosteric modulators of mGlu5 show functional selectivity i.e. they have the potential to selectively activate distinct downstream signalling pathway (Nickols & Conn, 2014). Here we investigated the possible role of the PI3K signalling, one of the pathways activated by group I mGluRs (Rong et al., 2003). Moreover, PI3K signalling leads to Akt activation, which is well established to have neuroprotective actions (Datta et al., 1999; Kandel & Hay, 1999).

Consistent with this, the PI3K inhibitor LY294002 completely abolished the neuroprotective effects of DHPG and VU0092273, suggesting the crucial role of the mGluR5-PI3K-Akt signalling pathway in their mechanism of action. Analysis of active phosphorylated Akt (pAkt) showed that pAkt was activated by VU0092273 but not the mGlu1 PAM VU0483605, indicating that activation of PI3K signalling pathway is mGlu5 selective. Moreover, the pro-apoptotic kinase GSK3 β (Chen et al., 2004; Linseml et al., 2004) a downstream target of pAkt (Pap et al., 1998; Datta et al., 1999; Yamaguchi et al., 2004), also showed increased phosphorylation. This is important because phosphorylated GSK3 β (pGSK3 β) is inactivated (Pap et al., 1998; Datta et al., 1999) so its inhibition can enhance cell survival.

Severe neurological insults trigger a 'molecular switch' that prevents GluA2 AMPA receptor subunit gene expression in cells destined to die (Pellegrini-Giampietro et al., 1997). The GluA2 hypothesis predicts that Ca²⁺ entry through GluA2-lacking AMPA receptors in neurons that normally express Ca²⁺-impermeable channels contributes to or causes delayed cell death in response to

endogenous glutamate (Pellegrini-Giampietro et al., 1997; Liu et al., 2006; Henley & Wilkinson, 2016; Koszegi et al., 2017).

We observed the GluA2 down-regulation 12 h after OGD injury, revealing that this phenomenon precedes cell death, which occurs 24 h later. Importantly, only mGluR5 PAM significantly prevents the GluA2 down-regulation induced by OGD injury. Moreover, when we measured GluA2 protein levels in presence of PI3K inhibitor, the prevention of GluA2 down-regulation was blocked when mediated by mGluR5 PAM, suggesting that the mGluR5 triggers the GluA2 up-regulation through the activation of PI3K signalling pathway, known to be also involved in activation of nuclear transcription factors, that regulate the expression of a large number of genes, critical for survival (Walton & Dragunow, 2000).

Thus, the mGluR5 exerts its neuroprotective effects against OGD injury by activating the PI3K signalling pathway, through which it triggers all the cascades which lead to block apoptotic processes and promote the cell survival, and enhances the GluA2 up-regulation, preventing the excitotoxic events which cause the delayed cell death.

Taken together our data demonstrate that in our OGD/recovery model mGlu5 (i) activates the PI3K-Akt pathway to promote survival; (ii) reduces GluA2 down-regulation which prevents the excitotoxic cascade triggered by the increased Ca^{2+} influx due to GluA2-lacking AMPA receptors; and (iii) contributes to reactive astrogliosis which can assist in rescuing post-ischemic neurons from OGD-induced cell death. Thus, we propose that selective activation/potentiation of mGlu5 signalling represents a promising strategy for the development of new interventions to reduce or prevent ischemia-induced neuronal death.

Acknowledgement

We would like to thank Dr. Jeffrey P. Conn (Vanderbilt University Medical Center, Nashville, TN, USA) for the generous gift of VU0483605 and VU0092273. This work was supported by grants from Ente Cassa di Risparmio di Firenze (ECRF 2015.0964) and the University of Florence. D.L. was a recipient of a post-doctoral fellowship from the University of Florence.

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Figure legends

Figure 1. Dose-dependent effects of DHPG against OGD injury in rat organotypic hippocampal slices. (A) Schematic diagram showing that slices were incubated with mGlu receptor agonists and antagonists during OGD and the subsequent recovery period. Twenty-four h later slices were incubated with PI for fluorescence detection of their optical density. (B) Hippocampal

slices, photographed under fluorescence optics, displaying background levels of fluorescence under control conditions, an intense PI labeling in the CA1 sub-region 24 h after exposure to 30 min OGD, a reduction in PI fluorescence when incubated with 1 μ M DHPG, that was prevented by the mGlu5 antagonist MPEP but not by the mGlu1 antagonist LY367385, and a marked increase in OGD-induced PI fluorescence when incubated with 300 μ M DHPG. (C) Quantitative analysis of OGD-induced CA1 PI fluorescence expressed as percentage of OGD toxicity. Incubation with 1 μ M DHPG significantly attenuated OGD injury; the effect was completely reverted by the co-incubation with the mGlu5 antagonist MPEP (10 μ M) but not with the mGlu1 antagonist LY367385 (100 μ M). Incubation with 10 μ M DHPG had no effect whereas 100 and 300 μ M DHPG exacerbated OGD injury. Values represent the mean \pm SEM of at least three experiments performed in quadruplicate. **P < 0.01 and *P < 0.05 vs. 30 min OGD alone (ANOVA + Tukey's w-test).

Figure 2. The mGlu5 receptor PAM VU0092273 but not the mGlu1 receptor PAM VU0483605 attenuates OGD injury in hippocampal slices. The experiments were carried out and drugs were added as shown in Fig. 1A. (A) Hippocampal slices, photographed under fluorescence optics, displaying selective damage of the CA1 subregion 24 h following 30 min OGD and a reduction in PI fluorescence when incubated with the mGlu5 receptor PAM VU0092273, that was reverted by the mGlu5 antagonist MPEP, but not with the mGlu1 receptor PAM VU0483605. (B) Quantitative analysis of OGD-induced CA1 PI fluorescence expressed as percentage of OGD toxicity. Incubation with 10 nM VU0092273 significantly attenuated OGD injury; the effect was reverted by co-incubation with the mGlu5 antagonist MPEP (10 μ M). The mGlu1 receptor PAM VU0483605 (1 μ M) incubated alone or in combination with the mGlu1 antagonist LY367385 (100 μ M) had no effect on OGD injury. Values represent the mean \pm SEM of at least three experiments performed in quadruplicate. **P < 0.01 vs. 30 min OGD alone (ANOVA + Tukey's w-test).

Figure 3. Effects of DHPG and VU0092273 on neurons, astrocytes and microglia in the CA1 area of organotypic hippocampal slices 24 h after exposure to 30 min OGD. Merge1-4: Representative confocal images of merged immunostaining of neurons (NeuN), astrocytes (GFAP)

and microglia (IBA1) in control (Merge 1), OGD (Merge 2), OGD+DHPG (Merge 3) and OGD + VU0092273 (Merge 4). The images shown in Fig. 3A1-A4 (neurons), 3B1-B4 (astrocytes), and 3C1-C4 (microglia) are obtained stacking 3 consecutive confocal z-scans (1 μm z-step each, total 3 μm) taken at 10 μm depth inside the slice. Images A1-C1a, A2-C2a, A3-C3a and A4-C4a are split images from a triple immunostaining performed on the same slice per experimental group. Scale bars: A1-A4, B1-B4, C1-C4: 100 μm . B1a-B4a, C1a-C4a: magnifications of the framed areas in the above panels. Scale bars: 15 μm . C3b-C4b: magnifications of the microglial cells pointed by the arrows in panels C3a and C4a, respectively. The dotted lines highlight the outlines of the microglial cells. Scale bars: 7.5 μm . D: NeuN fluorescence intensity of CA1 pyramidal neurons. $**P < 0.01$ vs. control (ANOVA + Tukey's w-test). E: GFAP fluorescence intensity in CA1. $*P < 0.05$ vs. control (ANOVA + Tukey's w-test). F: IBA1 fluorescence intensity in CA1. $**P < 0.01$ and $*P < 0.05$ vs. control (ANOVA + Tukey's w-test). Values on the y axes are ratios between positive pixels above threshold and total pixels of regions of interest and represent the mean \pm SEM of at least three experiments performed in quadruplicate.

Figure 4. The PI3K–Akt signaling pathway mediates the protective effects evoked by mGlu5 receptors. (A) The PI3K inhibitor LY294002 prevents the neuroprotective effects induced by mGlu5 activation. *Top:* Hippocampal slices, photographed under fluorescence optics, displaying increased PI fluorescence in the CA1 subregion 24 h after 30 min OGD and a marked reduction of neuronal injury when incubated with 1 μM DHPG and 10 nM VU0092273, that was partially prevented by 10 μM LY294002. *Bottom:* Quantitative analysis of CA1 PI fluorescence showing that OGD injury was reduced, in a manner that was prevented by 10 μM LY294002, when slices were co-incubated with 1 μM DHPG or 10 nM VU0092273. Bars represent the mean SEM of at least four experiments run in triplicate. $**P < 0.01$ and $*P < 0.05$ vs. 30 min OGD alone (ANOVA + Tukey's w-test). (B-C) Activation of mGlu5 receptors prevents the decrease in Akt and GSK3- β phosphorylation induced by 30 min OGD. Slices were exposed to the OGD for 30 min and processed for Western blotting 24 h later. The mGlu receptor PAMs VU0483605 (1 μM) and VU0092273 (10 nM) and the PI3K inhibitor LY294002 (10 μM) were added to the incubation

medium during OGD and the subsequent 24 h recovery period. *Top*: representative Western blots using polyclonal rabbit antibodies directed against phospho-Akt (Ser473) (B) or phospho-GSK3- β (Ser9) (C). The numbers on the right indicate the estimated molecular mass (kDa). α -Tubulin was used as loading control. *Bottom*: quantitative analysis of immunoreactive bands, showing that 30 min OGD induced a significant decrease in the phosphorylation of Akt (B) and GSK3- β (C), that was prevented by the mGlu5 PAM VU0092273 but not the mGlu1 PAM VU0483605. The PI3K inhibitor LY294002 almost completely reverted the effects of VU0092273. Data are expressed as percentage of control phosphorylation in control untreated slices. Bars represent the mean \pm SEM of three experiments. **P < 0.01 vs. control (ANOVA + Tukey's w test).

Figure 5. Effects of mGlu5 receptor activation on the expression of GluA2-lacking AMPA receptors in organotypic hippocampal slices exposed to OGD. The experiments were carried out and drugs were added as shown in Fig. 5A. (A) Schematic diagram showing that slices were incubated with drug during 30 min OGD and the subsequent recovery period of 12h. (B) Activation of mGlu5 receptors prevents the down-regulation of the GluA2 AMPA receptor subunit following OGD via the PI3K–Akt signaling pathway. Slices were exposed to OGD for 30 min and processed for Western blotting 12 h later. *Top*: A representative Western blot using a polyclonal goat antibody directed against GluA2. α -Tubulin was used as loading control. The numbers on the right indicate the estimated molecular mass (kDa). *Bottom*: quantitative analysis of immunoreactive bands, showing that 30 min OGD induced 12 h later a significant decrease in the expression of GluA2, that was significantly prevented by the mGlu5 PAM VU0092273. The PI3K inhibitor LY294002 reverted the effects of VU0092273. Data are expressed as percentage of GluA2 levels in control untreated slices. Bars represent the mean \pm SEM of three experiments. **P < 0.01 vs. control (ANOVA + Tukey's w test). #P < 0.05 vs. OGD alone. (C) NASPM attenuates OGD injury in a dose-dependent manner in organotypic hippocampal slices. Data are expressed as percentage of OGD-induced PI fluorescence in CA1 as observed 24 h after OGD. Values represent the mean \pm SEM of at least three experiments performed in quadruplicate. *P < 0.05 vs. 30 min OGD alone (ANOVA + Tukey's w-test).

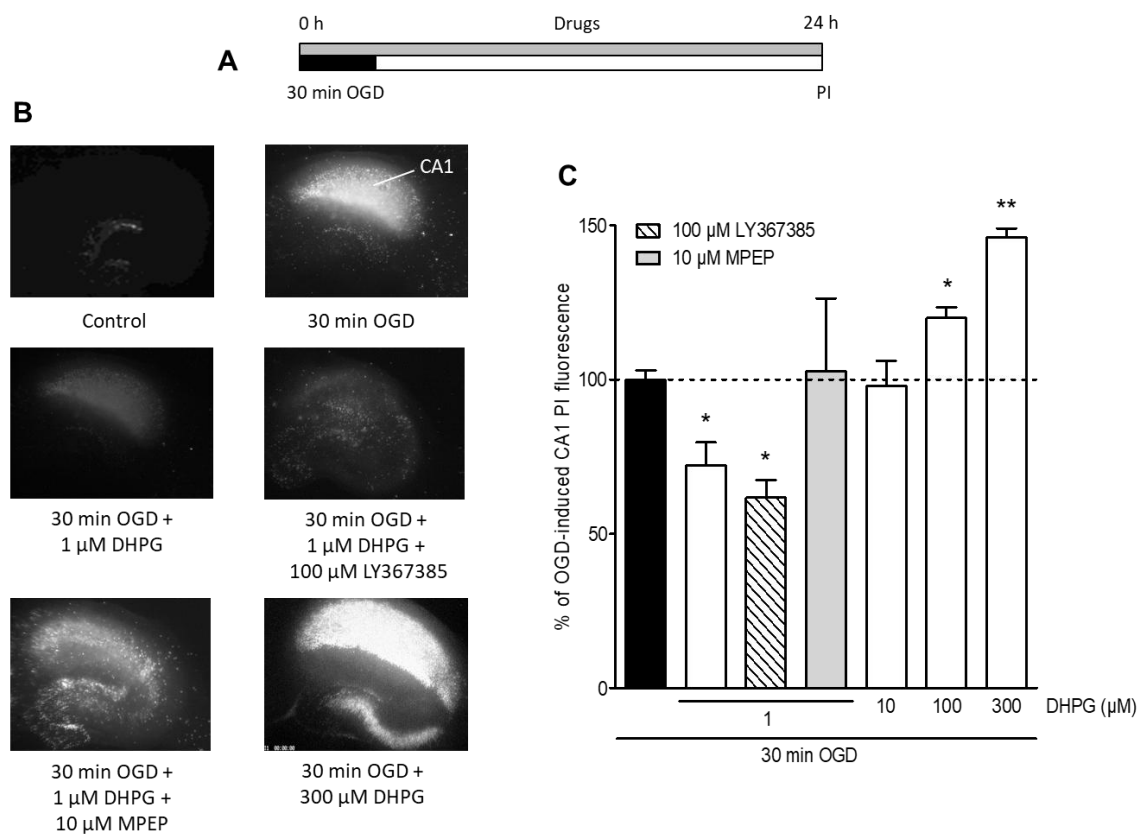


Figure 1

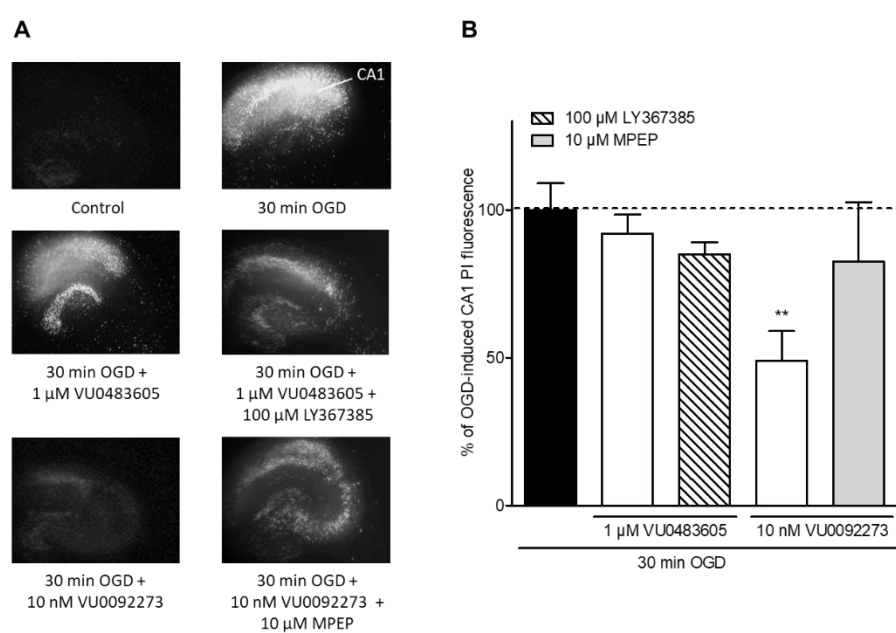


Figure 2

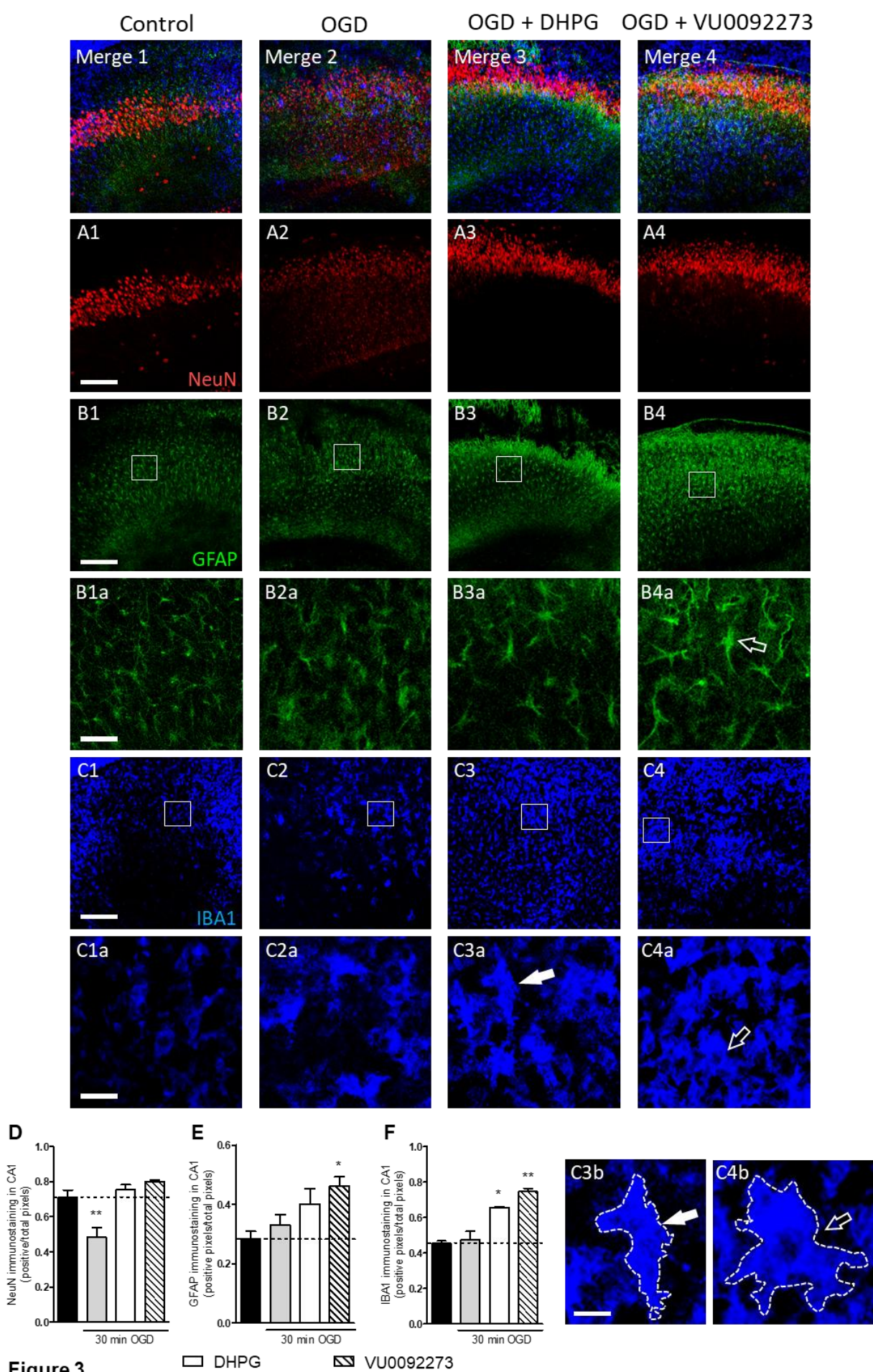


Figure 3

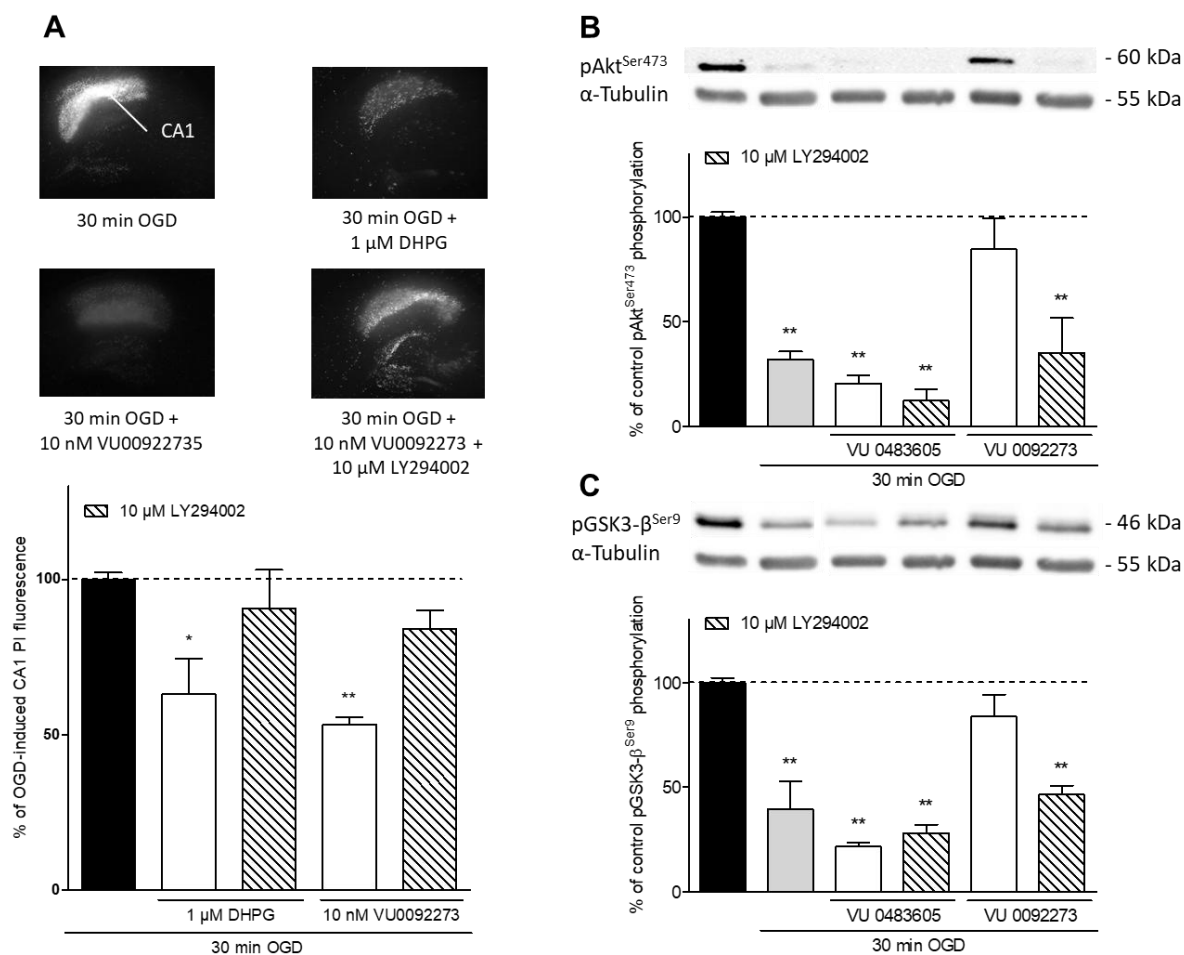


Figure 4

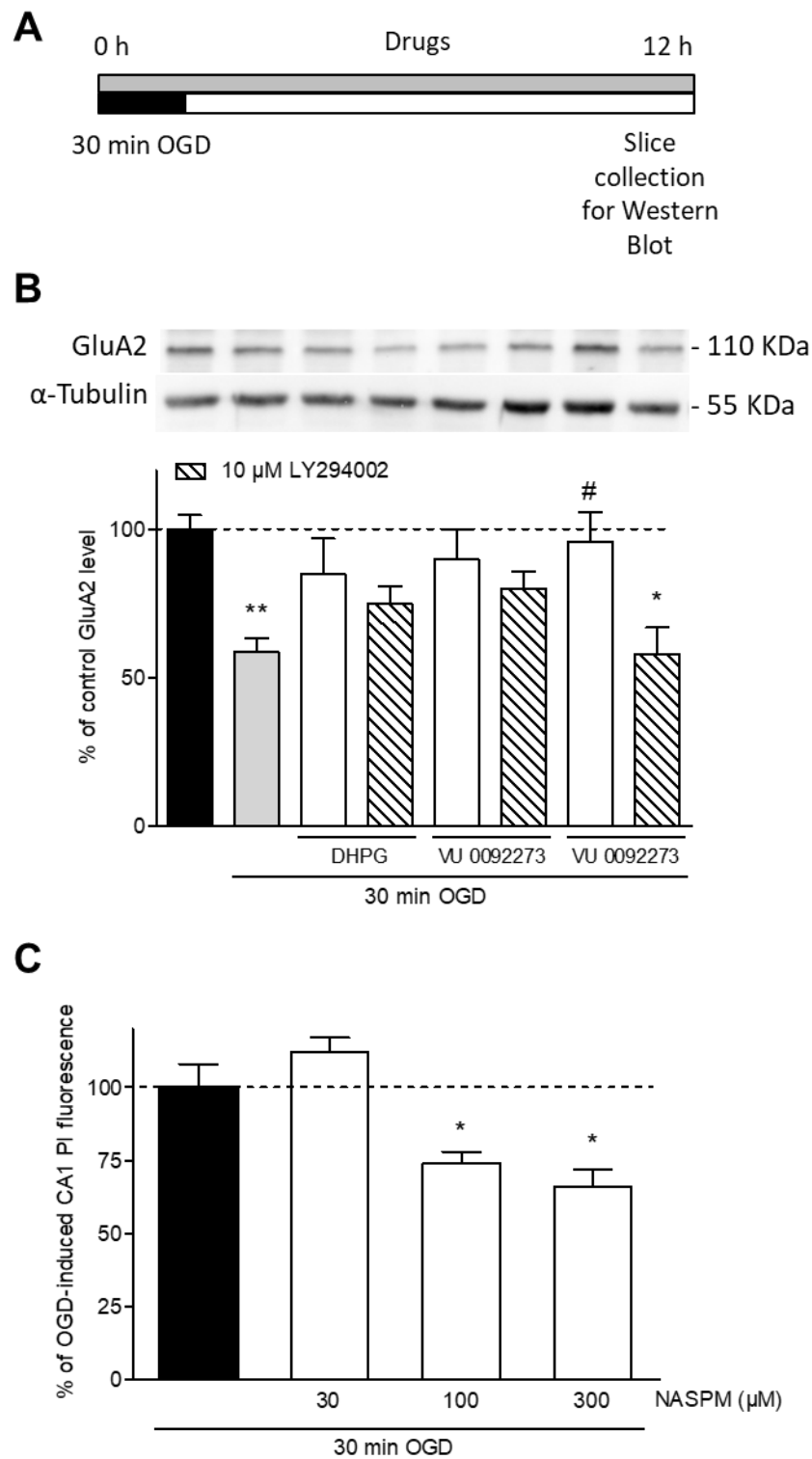


Figure 5

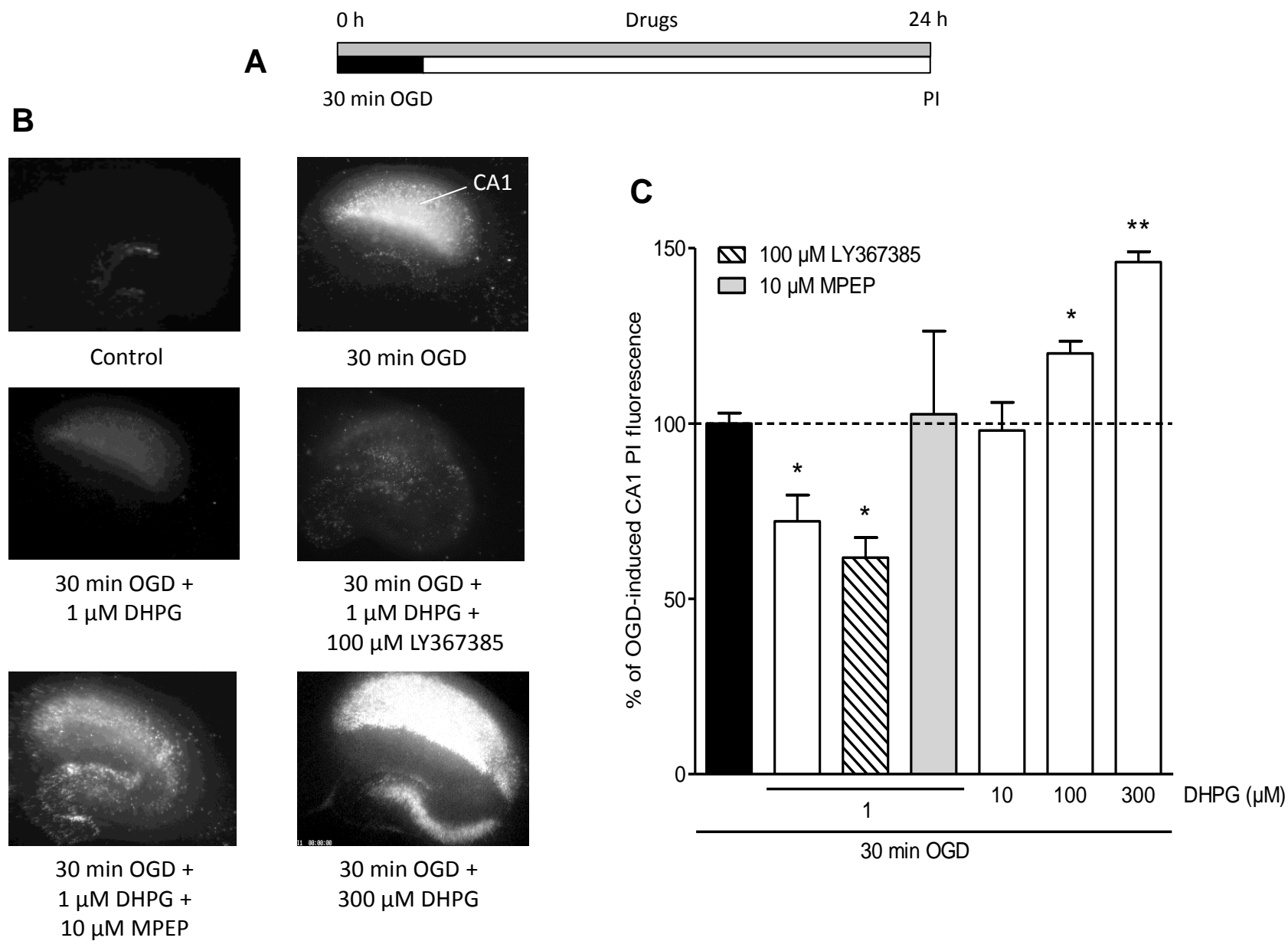
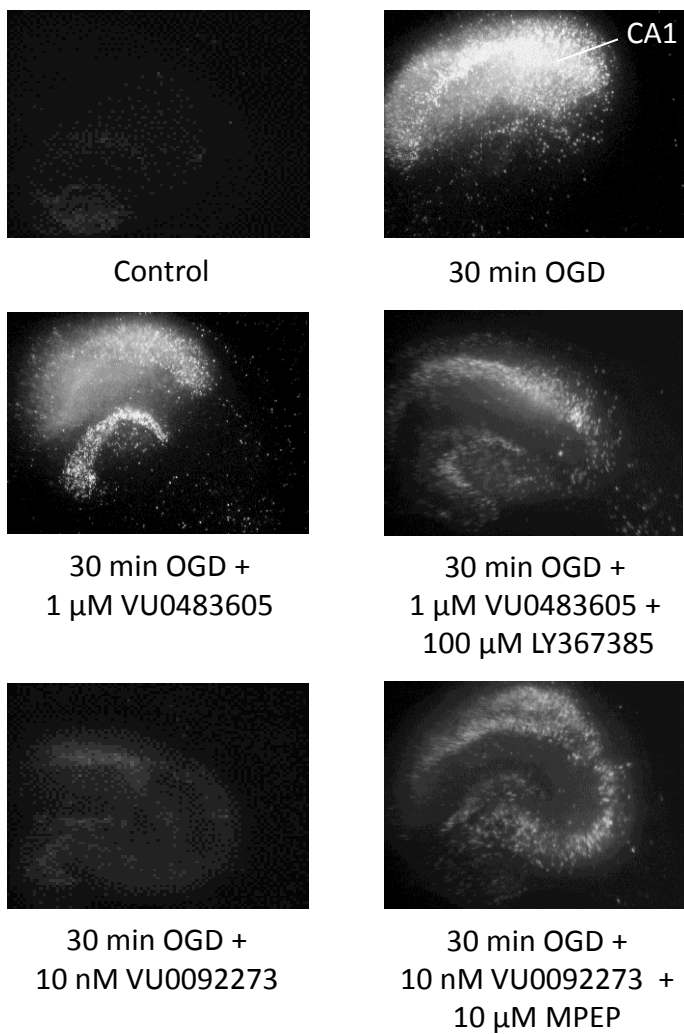
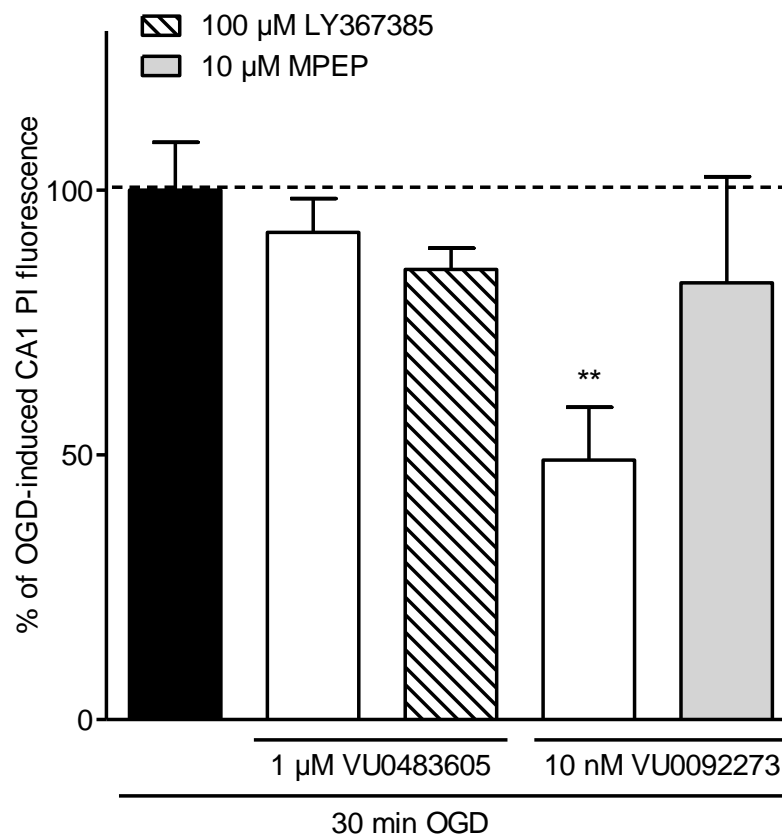


Figure 1

A**B****Figure 2**

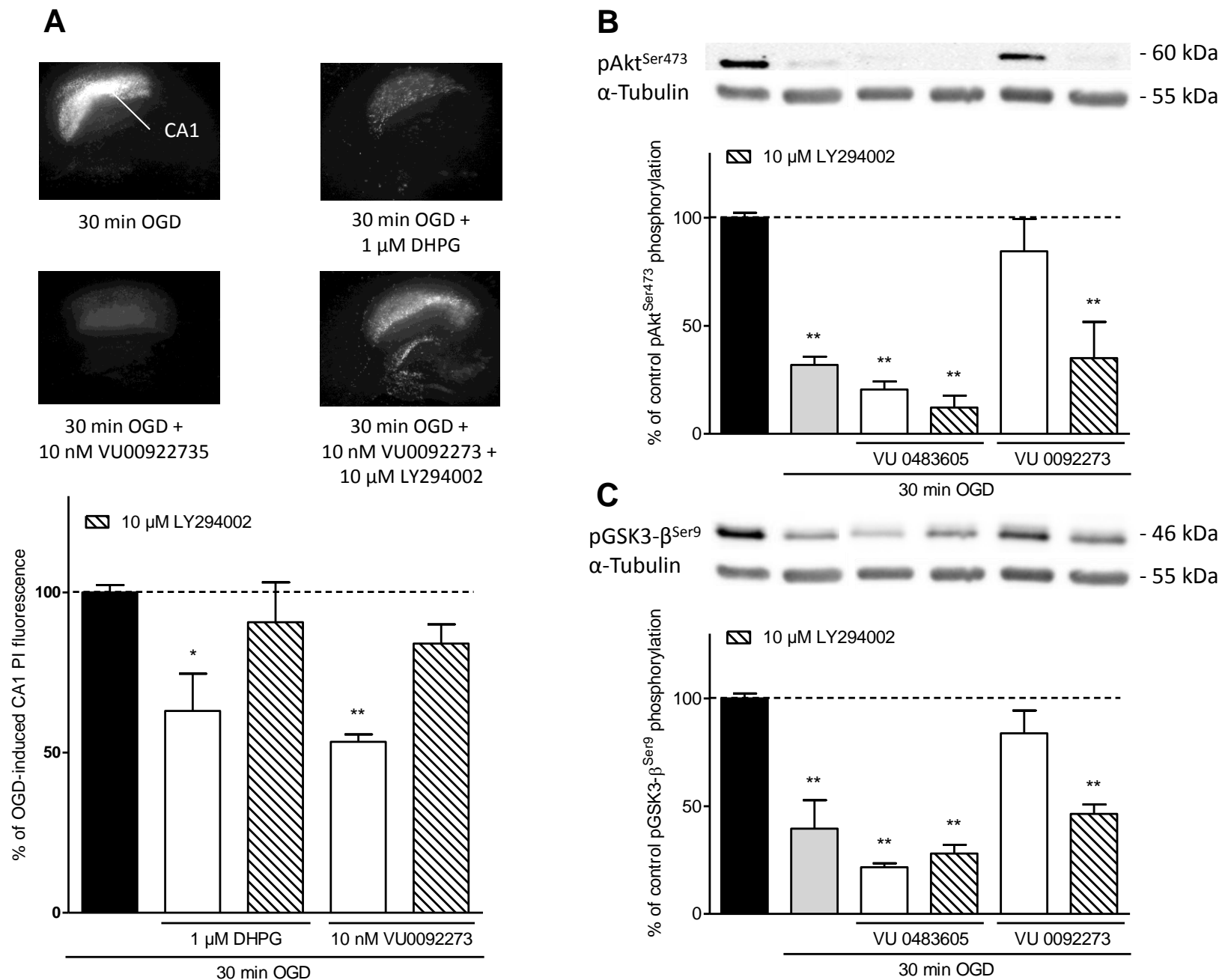


Figure 4

Figure(s)

